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REVIEW: The Application of Dendritic Cell-derived Exosomes in Tumour Immunotherapy

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Cancer arises from the aberrant proliferation of a single transformed cell. This population acquires the ability to metastasise. An effective way to remove cancer cells from the body is to activate tumour-specific cytotoxic T cells (CTL). Activation of naive T cells depends on the unique antigen presenting capacity of DC. Activated tumour antigen-specific CTL can destroy cancer cells without harm to normal tissue. Their ability to stimulate antigen specific T cell responses makes DC attractive candidates to potentiate anti-tumour immunity. Several studies have demonstrated the efficacy of DC based anti-tumour immunotherapy and the goal now is to optimise immune responses induced by DC, so that effective strategies in treating cancer may be realised. One way to do this is to identify DC characteristics which make them more effective in T cell stimulation. Another is to use exosomes, the antigen presenting vesicles secreted by DC, in order to induce potent anti-tumour immune responses. The non-cellular nature of exosomes offers several advantages for use in tumour immunotherapy.

CANCER

In the year 2000, an estimated ten million people worldwide will be diagnosed with cancer.[1] Of those stricken, more than half will eventually succumb to the disease. In fact, many developed nations of the world have seen an increase in the number of cancer-related deaths per capita since the early 1970s.[2] The underlying mechanism responsible for cancer formation is the accumulation of mutations in genes involved in regulating cell proliferation, survival and differentiation. This results in unregulated proliferation of the progeny of a single mutated cell. The process of carcinogenesis often leads to aberrations in tissue matrix proteases and adhesion molecules that are essential for keeping cells associated with their surrounding tissue. Such aberrations mean that cells are then able to invade the surrounding tissue and eventually spread to sites throughout the body. At this point, the transformed cell population has the definitive characteristics of cancer; unregulated growth and the ability to metastasize. As a result of the metastatic nature of cancers and their capacity to genetically change, each transformed cell can seed new tumour masses at sites quite distal from the primary focus of disease. Complete cure therefore requires the removal or eradication of all abnormal cells while preventing excessive harm to normal tissue. An attractive approach to achieve this would be to induce an adaptive immune response against a cancer. Such a response would monitor the whole body for tumour cells and target them for destruction. Furthermore, primed anti-tumour immune cells

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could generate a memory response which would be protective should cancer cells escape initial recognition leading to relapse of the disease.

Cancer Immune Recognition

Tumour recognition by T cells of the immune system, particularly by $CD8^+$ cytotoxic T cells (CTL), is central to tumour immunity. This has been demonstrated in studies showing that animals can be protected against tumour challenge through immunisation with T cells derived from syngeneic animals primed with tumour antigens.[3] Many isolated human tumour cells also bear specific tumour-associated antigens (TAA), which allow their recognition and destruction by autologous CTL *in vitro*.[4,5]

T cell receptors (TCR) on CTL mediate recognition of non-self antigens displayed as peptides associated with major histocompatibility class I molecules (MHC-I). Upon MHC-I/antigen recognition, CTL induce lysis and apoptosis of the antigen-bearing cell, by either releasing lytic granules on to the cell or via a Fas/Fas-ligand (Fas-L) interaction[6] (Fig. 1). TCR ligation also induces *de novo* synthesis of lytic granules allowing CTL to continue killing many target cells in succession.[7]

TAA recognized by CTL can be classified into five major groups based on the pattern of expression of their parent protein.[4,5] These include tumour-specific antigens generated from mutated genes, tissue-specific (but not tumour-specific) differentiation antigens, reactivated embryonic gene products, viral antigens expressed on virally-induced tumours and ubiquitous antigens, expressed on a range of normal cells. The specificity of viral and tumour-specific antigens make them the most promising targets for immunotherapy involving induction of cell-mediated immune responses.

Activation of Cytotoxic T Cells

The generation of anti-tumour effector CTL involves activation of naive $CD8^+$ T cells. Activation requires at least two signalling events.[8] Firstly, a primary signal must be provided by MHC-I/antigen interaction with TCR. Secondly, a costimulatory signal must also be provided by the cell bearing MHC-I/antigen complexes (Fig. 2). CD80 and CD86 are two costimulatory molecules on DC each of which

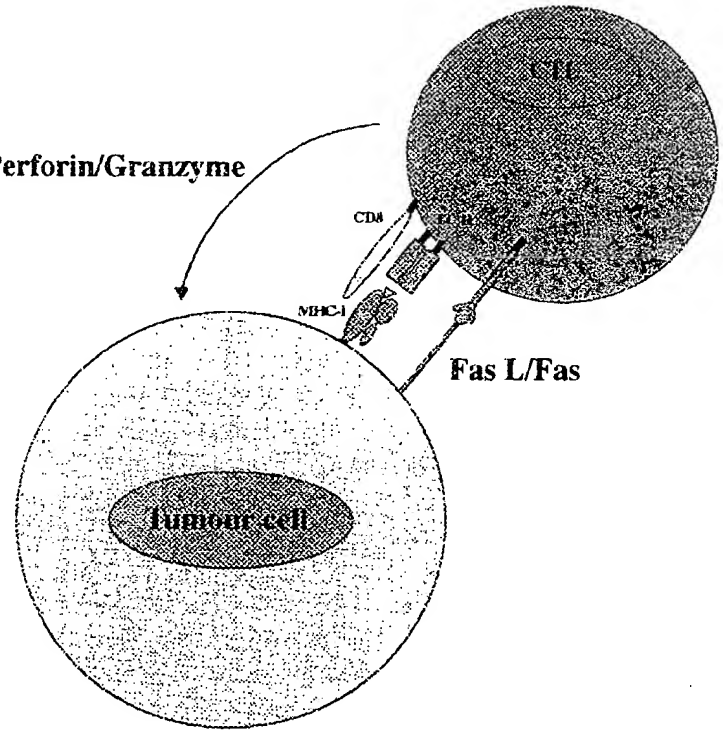

Fig 1. Cytotoxic T cells can destroy target tumour cells through induction of apoptosis. The function of CTL is to detect cells bearing non-self antigen displayed as peptides on MHC-I and eliminate them directly. Crosslinking of TCRs on CTL results in the polarised degranulation of preformed lytic granules containing perforin and granzymes A and B in the direction of cells bearing the crosslinking ligands (MHC-I/antigen). Perforin molecules then polymerise within the target cell membrane creating a hydrophilic pore. This can reduce the osmolarity of the cell leading to cell death as well as allow entry of granzymes which precipitate apoptosis of the targeted cell. Moreover, CTL can also trigger apoptosis through binding of their surface Fas molecules to FasL on target cells.

can bind to CD28 receptors on the naive $CD8^+$ T cells. Only with concomitant signalling via both MHC-I/antigen and costimulatory molecules will naive $CD8^+$ T cells be activated to differentiate into CTL.

In many instances, $CD4^+$ T helper type-1 (T_{H1}) cells are also required to potentiate full activation of naive $CD8^+$ T cells.[9] T_{H1} cells secrete cytokines including interleukin(IL)-2 and IL-7, which aid in promoting the proliferation and differentiation of $CD8^+$ T cells into CTL.[10] T_{H1} cells recognise antigens presented by MHC class II molecules (MHC-II). Before T_{H1} cells can act as effectors, they must themselves become activated. In this case, activation requires MHC-II/antigen-TCR signalling in conjunction with CD80/CD86-CD28 costimulator interaction on the naive $CD4^+$ T cell. Thus, optimal activation of tumour-reactive CTL requires MHC-I and costimulatory signals as well as effector T_{H1} cells.

The receptor requirements for CTL activation precludes a role for tumour cells as antigen presenting cells (APC) since they usually lack expression of costimulatory molecules and MHC-II. Instead, the immune system depends on specialized APC that express MHC-I, MHC-II and costimulatory molecules. APC can include macrophages, B cells and dendritic cells (DC). However, DC are thought to be the only APC capable of stimulating a naive T cell response making them potential candidates for induction of anti-cancer immunity in a clinical setting.[11]

Dendritic Cells

DC are characterized on the basis of morphology, expression of cell surface markers and functional capacity in stimulating T cell responses. Morphologically, DC are stellate in appearance with long cytoplasmic extensions or dendrites.[12] While there are no definitive cell surface markers for DC, they can be distinguished from other haemopoietic cells by the cell surface molecules which they express. These include CD80, CD86 and MHC-II, in both human and murine DC populations, as well as CD1a, CD83, CD58 in human and DEC-205 and CD11c in murine DC.[13] Functionally, all DC have the ability to present antigen and efficiently activate naive T cells. Despite common characteristics, there is evidence to suggest that all DC are not derived from a common progenitor. There may be multiple precursors including those of myeloid origin and those originating from a lymphoid progenitor.[14]

Evidence for a myeloid DC lineage is based on the ability of $CD34^+$ myeloid progenitor cells to develop into DC in the presence of the myeloid growth factor, granulocyte macrophage-colony stimulating factor (GM-CSF).[15,16] Support for a lymphoid DC lineage, is based on evidsence that DC can be propagated from thymus-derived $CD4^{low}$ T cell precursors in the absence of GM-CSF. This distinguishes them from cells which are precursors of myeloid DC [17]. The ability of DC to differentiate along distinct pathways has led to the hypothesis that DC of the myeloid and lymphoid lineage have distinct functional properties.[18] Thymic lymphoid-related DC play an essential role in clonal deletion of potentially autoreactive T cells during negative selection in the thymus.[19] This has led to the proposal that lymphoid-related DC maintain immune tolerance in both primary and secondary lymphoid tissues. Myeloid DC are postulated to be involved primarily in T cell activation. However, it is not yet clear whether these distinct functions reflect DC derived from different lineages or DC of a single lineage able to attain different functional capacities.

Antigen Contact, Capture and Processing

Both immature and mature stages in DC development have been described. Immature DC, lack high expression of MHC-II, CD80 and CD86 molecules and are therefore poorly immunogenic.[20] These cells are specialised for contact, uptake and processing of antigen for presentation on MHC-I/II molecules. Immature DC are located throughout the body, including skin and mucosal epithelia (Langerhans cells), the interstitial spaces of most non-lymphoid tissues (interstitial DC) and within primary and secondary lymphoid organs (lymphoid DC).[21] This allows them to engage antigen throughout the whole body.

Immature DC are able to take up antigen by phagocytosis, absorptive endocytosis and pinocytosis.[11] DC are now known to express a range of receptors for absorptive endocytosis, including the mannose receptor, the putative glycosylated antigen receptor, DEC-205, and Fc receptors which take up

opsonised antigen.[22,23] DC also express $\alpha_v\beta_5$ intergrin and CD36 which are thought to mediate the specific uptake of apoptotic bodies.[24] This represents a mechanism by which intracellular antigens released from apoptotic cells are presented to T cells. In addition, DC have the capacity to engulf large amounts of extracellular fluid through macropinocytosis, allowing soluble antigen uptake.[22] Protein antigens taken up by DC must be processed into peptide fragments before they can be presented to T cells on MHC-I or MHC-II. Newly synthesised MHC-II associate with a chaperone-like molecule, the invariant chain (Ii) in the endoplasmic reticulum (ER).[25] Ii contains endosomal sorting signals allowing traffic of MHC-II/Ii complexes to the endosomal pathway where they meet antigen.[26] Protein antigens in the endosomal pathway are degraded into peptides through proteases, including cathepsin D, which are activated by the low pH environment within acidic endosomes.[27] Peptides can then associate with MHC-II in a process catalysed in humans by human leucocyte antigen (HLA)-DM.[28] In DC, the processing of antigens for MHC-II association is enhanced by the presence of endosomes highly enriched in MHC-II. These are the MHC-II rich compartments.[22,29] Once associated with peptide, MHC-II acquire a stable conformation, allowing their transport to the cell surface.[30]

MHC-I antigen loading occurs primarily in the endoplasmic reticulum (ER).[31] Cytoplasmic proteins are the recognised source of peptides for MHC-I presentation. Proteins are degraded in the cytoplasm via a proteinase complex, the proteosome.[32] Peptides can then be transported into the ER lumen by ATP-dependent transporters associated with peptide presentation (TAP).[33] Within the ER, chaperones such as calnexin, assist peptides to associate with MHC-I.[34] MHC-I/antigen complexes are then transported directly to the plasma membrane (PM) without intersecting the endosomal pathway.[35] In addition to processing cytoplasmic-derived antigens, DC also have the capacity to process exogenous antigens for MHC-I presentation. It has been found that extracellular antigens sequestered in endosomes can be transferred to the cytosol.[36] Antigens are then postulated to follow the proteasome/TAP dependent pathway of MHC-I loading in the ER. In support of this, DC can not present extracellular antigens on MHC-I if they are TAP deficient or treated with proteasome inhibitors.[37,38]

Maturation, Migration and T Cell Activation

Mature DC are highly immunogenic and express high levels of MHC-I/II and costimulatory molecules including CD40, CD80 and CD86.[11] Maturation of DC can be initiated by cytokines associated with inflammation, including tumour necrosis factor-α (TNF-α), bacterial products such as lipopolysaccharide (LPS)[39] and the uptake of pathogen-associated antigens.[40] Maturing DC downregulate antigen uptake capacity by lowering their expression of antigen receptors and their ability for macropinocytosis.[22] They also increase their production of MHC-I and II[40] and begin to discharge MHC-II/antigen complexes from MHC-II rich compartments to the cell surface.[41] Activated DC begin to migrate specifically to the T cell areas of local secondary lymphoid organ, the major site for inducing a primary immune response.[20] Migration is thought to involve DC expression of CCR7, a receptor specific for chemokines secreted by cells localised in lymphoid T cell areas.[42]

Mature DC express a novel chemokine, DC-CK1, that preferentially attracts naive ($CD45RA^+$) T cells.[43] They also express high levels of adhesion molecules including CD58, CD54 and CD11a.[11] These properties allow DC to attract and cluster with naive T cells, allowing MHC/antigen selection of antigen-specific T cells and their subsequent activation through costimulation. The immunogenicity of DC is further enhanced through secretion of T cell growth and activation factors, including IL-7 and IL-12[11] (Fig. 2). $CD4^+$ T cells respond to activation by increasing surface levels of CD40 ligand (CD40L),[10] which in turn binds to CD40 expressed on mature DC. This results in upregulation of CD80 and CD86 on DC.[20] Furthermore, CD40/CD40L interaction also empowers DC to directly activate naive $CD8^+$ T cells into CTL, bypassing the need for direct spatial interaction with T_{H1} cells.[44]

DC-derived Exosomes

In addition to direct interaction and secretion of cytokines, DC are also able to trigger T cell responses through the production of exosomes.[45,46] Exosomes are derived from MHC-II rich compartments within DC. In immature DC, these have been found to resemble lysosomes. They represent acidic

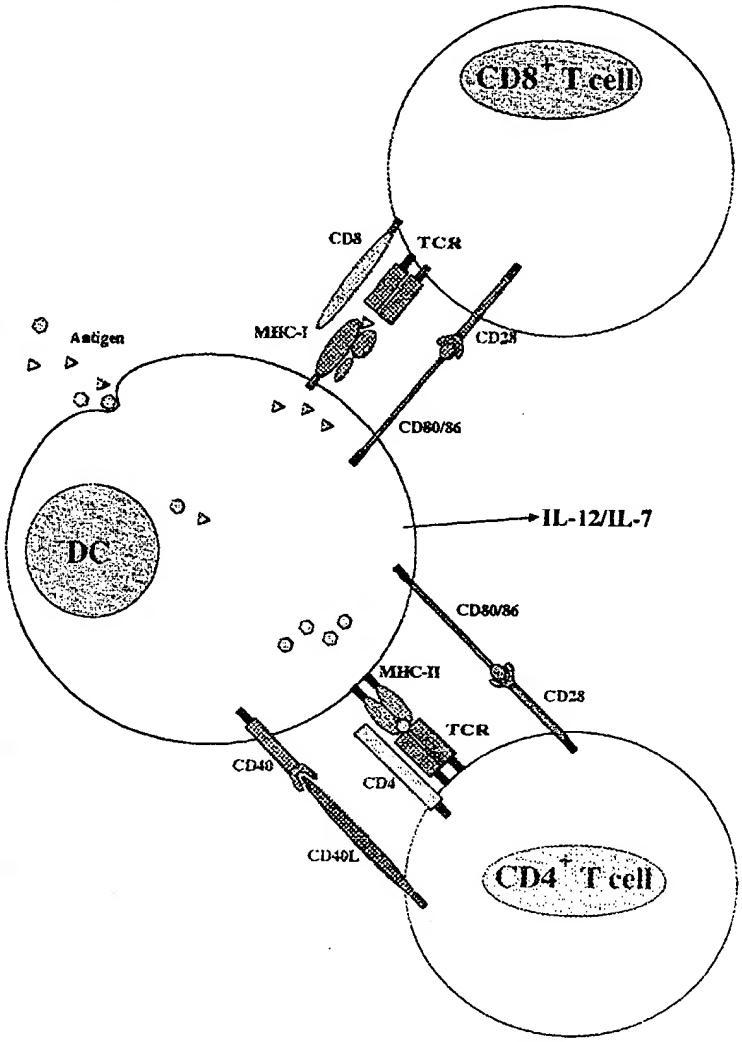

Fig 2. Mature DC express high levels of MHC-I/II and costimulatory molecules making them potent T cell activators. Mature DC express high levels of MHC-I and MHC-II molecules allowing them to select both $CD4^+$ T cells and $CD8^+$ T cells. Mature DC activate antigen-specific T cells through the expression of costimulatory molecules, including CD80 and CD86, as well as T cell growth/activation factors, including IL-12 and IL-7. In turn activated $CD4^+$ T cells expressing CD40L can enhance DC function by crosslinking CD40.

compartments late in the endosomal pathway and express lysosome-associate membrane proteins (LAMP).[47] In other APC as well as DC, some MHC-II rich compartments have been found to contain many intraluminal membrane vesicles.[45,46,48] These MHC-II rich compartments have been described as multivesicular endosomes. The internal vesicles within multivesicular endosomes, are thought to be formed by the inward budding of the limiting membrane of the endosomes.[49] Recent studies in human B cells have demonstrated that multivesicular MHC-II rich compartments can fuse with the PM, resulting in the release of intraluminal vesicles into the extracellular environment.[48] These exocytosed vesicles, or exosomes, express MHC-II and are able to specifically stimulate T cell proliferation. One study has also demonstrated that DC propagated in exogenous cytokines also secrete exosomes.[45,46] Exosomes are thought to be released from cells after fusion of multivesicular MHC-II rich compartments with the PM. In addition to expressing MHC-II, DC-derived exosomes have been shown to express MHC-I and the costimulatory molecule, CD86. Furthermore, exosomes derived from antigen pulsed DC can activate allogeneic T cells and also trigger MHC-I restricted responses from $CD8^+$ T cell clones *in vitro*. However, these responses can be much weaker than those generated by the DC from which they were derived.

Tumour Escape from Immune Recognition

Given the tremendous potential for DC to take up antigens and trigger T cell responses, one must question why tumour development ever occurs. Since tumours bear unique TAA, DC should be able

to process these and stimulate an immune response. However, the ability of tumours to acquire genetic variability endows tumour cells with enormous potential to avoid immune recognition.[50] Tumour cells have been demonstrated to alter peptide epitopes presented by MHC-I and to downregulate MHC-I/antigen surface expression.[51] Recent studies have shown that DC isolated from tumour-bearing animals have reduced antigen presenting capacity.[52] Tumours also produce IL-10, and transforming growth factor-β (TGF-β) which downregulate CTL activity.[53] Interleukin-10 and TFG-β secreted by a range of human tumours can inhibit DC ability to activate CTL.[54] Furthermore, IL-10 has been shown to prevent DC maturation and can result in DC presenting antigen in a tolerogenic manner.[55]

The growth of tumours in animals can also have more fundamental effects on DC function. Since tumours arise from outgrowths of endogenous tissue, it is unlikely that they are associated with release of inflammatory cytokines or other factors which might induce DC maturation.[56] Since maturation is required for DC to present antigen along with costimulatory molecules, it is unlikely that T cells will be directed against tumours during the early stages of tumour development. Tumour cells can, however, express high levels of anti-apoptotic factors such as FLIP, which reduces CTL-mediated apoptosis.[57] These factors all help to prevent tumour recognition by CTL.

An attractive way to counteract the lack of T cell stimulation would be to expand and manipulate patient-derived DC *ex vivo* and to deliver them back into the patient. These would then be capable of presenting tumour antigen in a manner which is stimulatory to T cells.

DC and Exosomes in Cancer Immunotherapy

There are now several techniques for generating large numbers of human DC. These involve either purification of immature DC precursors from peripheral blood,[58] or propagation of DC from peripheral blood monocytes or $CD34^+$ bone marrow (BM) progenitors using cytokines, including GM-CSF.[59] These DC have capacity to take up exogenous antigens and prime antigen-specific T cell responses. The potential exists for isolating DC from tumour-bearing patients, which can be pulsed with exogenous tumour antigens and reinfused back into the patient to stimulate immunity. In fact, murine studies using similar protocols have demonstrated that anti-tumour immunogenicity can be generated in this way. Vaccination of BM-derived DC pulsed with unfractionated whole tumour lysate can protect mice from lethal doses of tumour cells and markedly suppress the growth of established, poorly immunogenic tumours.[60] Similar responses have also been demonstrated using BM-derived DC pulsed with unfractionated acid eluted tumour peptides.[61] When presented on DC, purified tumour-specific peptides, including peptides of mutant p53, a commonly mutated gene in tumours, can also generate protective and therapeutic immunity against tumours in mice.[62]

The successful use of DC-based anti-tumour immunotherapy in murine studies has led to clinical trials in humans. In the first reported clinical trial, Hsu and colleagues[63] employed autologous peripheral blood-derived DC for the treatment of patients with B cell lymphoma. DC were pulsed with tumour-specific immunoglobulin (idiotypic) antigens. Several intravenous administrations of antigen-pulsed DC resulted in the development of measurable tumour-specific T cell responses in all patients. Two out of the four treated patients treated showed regression of tumour burden and one underwent complete remission. Clinical trials have also been carried out on patients with malignant melanoma.[64] Autologous DC, propagated from peripheral blood monocytes cultured in GM-CSF and IL-4, were pulsed with purified melanoma associated peptides or autologous tumour cell lysate. Patients received several injections of pulsed DC. Some peptide-specific CTL responses were detected and five incomplete and two complete remissions were observed amongst sixteen patients.

DC-derived exosomes have recently been characterised for their potential to protect and treat both poorly immunogenic tumours and highly aggressive murine tumours.[45] BM-derived DC cultured in GM-CSF and IL-4 and pulsed with either tumour-specific epitopes or unfractionated acid eluted tumour peptides, were used as the exosome source. Exosomes used to vaccinate mice against challenge with the aggressive mastocytoma, P815, could protect up to 75% of mice from disease. Furthermore, exosomes from BM-derived DC pulsed

with unfractionated acid eluted tumour peptides were able to induce remission in 40-60% of mice bearing established tumours. These mice maintained disease-free survival and had detectable tumour-specific CTL responses.[45] Parallel studies using tumour peptide-pulsed BM-derived DC revealed that exosomes were far more potent in treating mice with established tumours.

Conclusion

The potency of both DC and exosomes in generating a tumour-specific immune response warrants further investigation into their use as anti-tumour immunotherapeutic vectors. While it is clear that DC are potent stimulators of anti-tumour immune responses, very few studies have addressed how these responses can be optimised to completely eliminate tumour growth. DC isolation procedures for adoptive transfer need to take into account the lineage and maturation characteristics of DC and their capacity to be effective as an immunogen. It is anticipated that the response generated by DC can be optimised through identification of cell subsets through expression of immunostimulatory markers. Since various activators can upregulate expression of immunomodulatory markers, activated DC may be needed to gain tumour remission. In a clinical setting, use of modulated DC would entail isolation, tumour antigen pulsing and treatment of autologous DC *in vitro* to enhance stimulatory capacity. DC could then be reinfused back into the patient to bring about immunity. However, upon reinfusion, DC might be influenced by 'anti-maturation' factors like IL-10 and TGF-β secreted by tumours,[50] resulting in downregulation of the stimulatory capacity of DC and possibly anergy of tumour reactive T cells.[55] In this situation, exosomes, being non-cellular and therefore unaffected by inhibitory cytokines, may offer an advantage over DC in immunotherapy. Autologous DC would be pulsed with tumour antigens and treated with factors to increase production of stimulatory exosomes. Exosomes would then be isolated from DC and infused back to the patient. These exosomes being refractory to tumour influence could then stimulate tumour immunity.

There have only been two published reports to date of DC-derived exosomes.[45,46] Their potent capacity to trigger anti-tumour immune responses indicates that exosomes may provide a valuable vector for tumour immunotherapy. However, there has been very little characterisation of DC-derived exosomes and their potential in immunotherapy. While they are produced by DC after *in vitro* culture, it is not yet possible to determine whether they are produced *in vivo* and whether they play any significant role in immunity in the animal. Recent studies have shown that exosomes are produced in greater numbers from immature DC.[45] This is a concern since procedures currently used to amplify DC *in vitro* using cocktails of cytokines, give rise to cells resembling mature DC. What is needed is a continuous line of DC which are immature and which secrete exosomes. A long term culture (LTC) system has been described which generates DC in the absence of exogenous cytokines.[65-67] This system produces a continuous supply of immature DC and has recently been shown to be a valuable source of immunogenic exosomes (Quah and O'Neill, unpublished data). It is anticipated that LTC-DC, being relatively immature cells, will provide a valuable source of exosomes for study. The difficulty of isolating exosomes makes it important to establish a ready source of DC for experimental studies on exosome production and function.

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